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13. ABSTRACT (Maximum 200 Words) In order to better understand the role of estrogen metabolism as it relates to breast cancer etiology, a new analytical technique that can measure CE and CE-DNA adducts at low endogenous levels is being developed. This new technique is based on HPLC analysis of fluorescent probes specific for CE and CE-DNA adducts. An extraction procedure employing a solvent mixture of chloroform, dimethylformamide, and acetic acid was developed to extract CE-DNA adducts, CE, and MPEM from rat breast tissue. This extraction procedure involves homogenization of the tissue in 20 mL of the solvent mixture, removal of the solvent, followed by HPLC analysis. Reaction of α,α -dibromomalonates occurs quickly with catechols, and this malonate system is being developed to produce fluorescent probes for HPLC analysis. Reaction of diethyl α,α -dibromomalonate with catechol results in ketal formation. The resulting ethyl ester is tranesterified to incorporate anthracene groups. A one-step process using α,α -dibromomalonamides is also under development. The aim of this work is to develop new biomarkers for the early detection of breast cancer.				
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INTRODUCTION:

The metabolism of estrogen to procarcinogenic catechols has been hypothesized as an initiation step in the development of breast cancer [1-4]. Specifically, the over expression of 4-hydroxylase activity has been observed in organs prone to estrogen-induced tumors [5-8]. The relationship between increased production of 4-hydroxyestradiol (4-OHE₂) and 4-hydroxyestrone (4-OHE₁) metabolites with the increase occurrence of estrogen-induced tumors is still not clear. Hypothesis regarding redox cycling [9-13] and oxidation to electrophilic quinones have been examined [3,4]. Since oxidation of catechol estrogens (CE) to catechol estrogen quinones (CE-Q) has been shown to lead to CE-DNA adducts [4], we seek to develop an analytical technique that can measure these adducts at endogenous levels. The purpose of developing this assay is to examine whether CE-DNA adducts are present in breast cancer tissue. This would be a first step in understanding the etiology of breast cancer as it relates to estrogen metabolism, specifically, the role of 4-hydroxylase activity in the increase occurrence of estrogen-induced cancers. The scope of this method development involves the production of fluorescent probes, specific for the catechol moiety, so that low-level detection of these adducts can be accomplished. With the proper development of fluorescent probes, this assay would be very sensitive and selective towards CE-DNA adducts.

BODY:

Work relating to Task 1, "Develop an extraction procedure for the isolation of CE-adducts, CE and MPEM from rat mammary tissue", has been nearly completed. CE-DNA adducts, CE, and monophenol estrogen metabolites (MPEM) were added to ca. 0.5 g of rat mammary tissue (Sprague Dawley). The compounds were applied to the tissue in dimethylformamide (DMF) solutions and allowed to stay in contact with the tissue for one hour at room temperature. The tissue was then homogenized in a 50 mL centrifuge tube for 2 minutes using 20 mL of an optimized solvent mixture. The solvent mixture consisted of 55:40:5, chloroform, DMF, and acetic acid, respectively, containing 0.5g-per 100mL ascorbic acid. This solvent mixture has several advantages: 1) by employing greater than 50% chloroform, the mixture is non-flammable and can be used by conventional, metal-based homogenizers; 2) the chloroform-DMF mixture extracts both polar CE-DNA adducts, and the less polar MPEM; 3) the acetic acid, in combination with added ascorbic acid impedes air oxidation of catechol metabolites. Thus, 4-OHE₁-1-N7Gua (CE-DNA adduct), 4-OHE₁ (CE), and 4-methoxyE₁ (MPEM) were placed on the rat tissue in 20μL of DMF containing 100 nmol of the respective compound (see Figure 1 for metabolite structures). The tissue was homogenized for 2 minutes using 20 mL of the solvent mixture. The suspension was then centrifuged, the solvent decanted, and the decanted solvent was removed by reduced pressure evaporation. The residue was then re-dissolved into 100 μL of clean DMF and a 50μL aliquot was injected on a HPLC employing a C-18 reverse phase column. The compounds were eluted using a gradient of 0.5 percent acetic acid and methanol and detected by UV absorbance (280 nm). Using standards for calibration curves, recoveries for all compounds were assayed at 80 to 90%.

In order to test this methodology on smaller samples sizes, i.e. the low picomolar range to high femtomolar range, completion of Task 2, "Develop an HPLC analytical procedure, via pre-column fluorescence derivatization, for the femtomolar detection of CE-adducts, CE and MPEM in human breast tissue", needs to be finalized. The progress on Task 2 is discussed below.

Development of a fluorescent probe specific for the catechol moiety has been approached by developing structures that can react with the vicinal hydroxy groups selectively (see Figure 2). Since the vicinal hydroxy groups can act as nucleophiles, structures containing activated geminal dihalides have been synthesized and shown to react with catechols forming a spiro, ketal derivative. Several dihalide candidates have been developed (Year 1 and Year 2 report) with α,α-dibromomalonates showing the most promise (Figure 3). Work this past year on Task 2 has involved the synthesis of α,α-dibromomalonate esters containing anthracene and pyrene moieties, reaction of these new esters with catechol, and attachment of the anthracene fluorophore after the formation of the ketal derivative. In

addition, the synthesis of α,α -dibromomalonamides containing the anthracene fluorophore has recently been completed.

The synthesis of bis-(9-methylenanthryl) α,α -dibromomalonate was conducted using several methods; however, the reaction of this probe with catechol proved unsuccessful. Unlike the diethyl analog (Figure 3), the dianthryl probe produced 9-anthrylmethanol as the sole product in nearly quantitative yield (Figure 4). Several variations of solvent, base, and reaction temperature were tried with no success. Bis-(1-butylpyrene) α,α -dibromomalonate was also synthesized, but reaction with catechol again produce only alcohol side products. One possible difference for the reaction of the ethyl ester compared with esters containing anthracene or pyrene groups is the propensity for both anthracene and pyrene towards bromination. Dibromomalonates have been employed as brominating reagents [14].

To try to circumvent possible bromination issues, a strategy of first attaching the catechol ring to the ethyl ester, followed by hydrolysis, and then re-esterification via a nucleophilic substitution was conducted (Figure 5). This sequence avoids exposure of the labile anthracene group to the α,α -dibromomalonate reagent. This procedure did prove successful in generating a fluorescent adduct but suffers from undesirable experimental procedures. First, this derivation scheme involves three chemical steps as apposed to one step when using α,α -dibromomalonates directly. Second, the final ketal product elutes close to the 9-bromomethylantracene reagent. This reduces sensitivity since this reagent would be used in large excess when assaying biological samples. Third, the hydrolysis conditions using cesium hydroxide could result in reaction with other functional groups present on both CE-DNA adducts and CE. The application of this process to actual adducts is still ongoing.

The production of amide analogs of α,α -dibromomalonates is under investigation in order to produce a "one reaction" derivation procedure. The dibromomalonamides would be less reactive brominating reagents and may avoid the undesirable side products generated by the ester fluorophores. To test the reactivity of the amide analogs, bis-(N-phenyl) α,α -dibromomalonamide was synthesize and reacted with catechol (Figure 6). This reaction produced the ketal product in yields similar to diethyl α,α -dibromomalonates (Figure 3). Recently, the synthesis bis-(N-9-methlyanthranyl) α,α -dibromomalonamide has been completed (Figure 7). The reaction of this new probe with catechol is currently under investigation.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Extraction conditions for isolation of CE-DNA adducts, CE, and MPEM from rat breast tissue have been established.
- 2) Derivation procedure for fluorescence labeling of catechols has been developed using a three-step sequence of ketal formation, hydrolysis, and ester formation.
- 3) The synthesis of a dianthryl α,α -dibromomalonamide probe has been conducted.

REPORTABLE OUTCOMES:

- 1) Poster entitled, "Synthesis Of Fluorescent Probes Specific For The Catechol Moiety" was presented at the annual national spring meeting of the American Chemical Society, San Diego, CA, April 1, 2001.
- 2) Nicole Burns, an undergraduate research assitant, prented a talk at the Nebraska Academy of Sciences annual meeting entitled, "Synthesis of Fluorescent Probes Specific For The Catechol Moiety", Lincoln, NE, April 27, 2001.

- 3) Two Undergraduates, Stacy Hill and Nicole Burns, were employed full time during the summer of 1999, part-time during the academic year 2000/2001, and full time during the summer of 2001. Nicole Burns received her Bachelors Degree in May 2001 and will continue her studies in breast cancer research at the University of Nebraska Medical Center as a graduate student.

CONCLUSIONS:

The development of an extraction procedure capable of isolating CE-DNA adducts, CE, and MPEM from rat breast tissue has been advanced. This process is the main component of Task 1 and the only remaining issue to be addressed is performance on a smaller scale. The extraction procedure is fast and amendable to the analysis of several samples using standard laboratory equipment. This procedure should translate to the isolation of estrogen metabolites from human tissue.

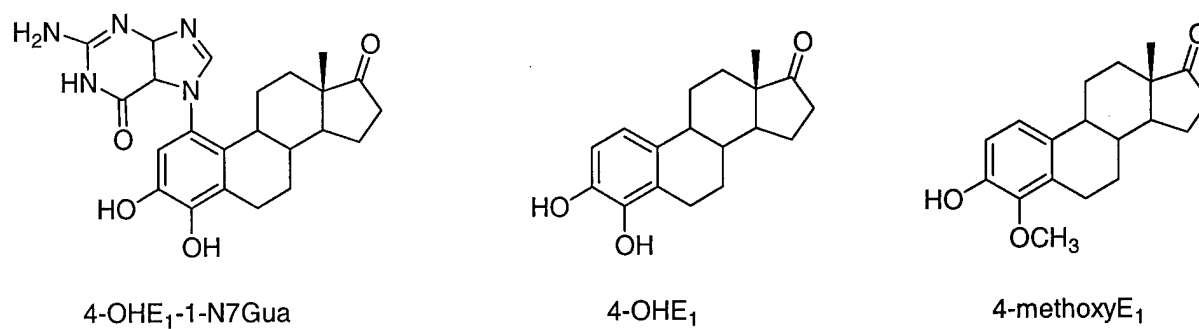
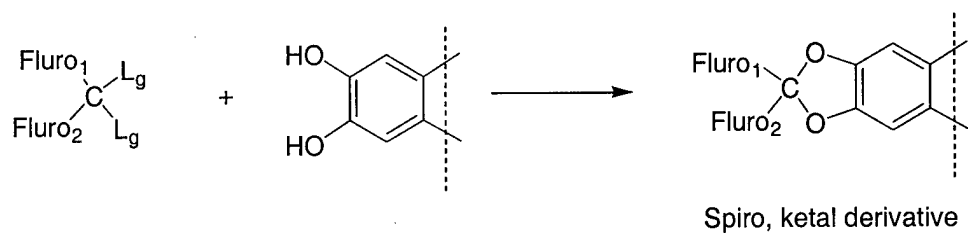
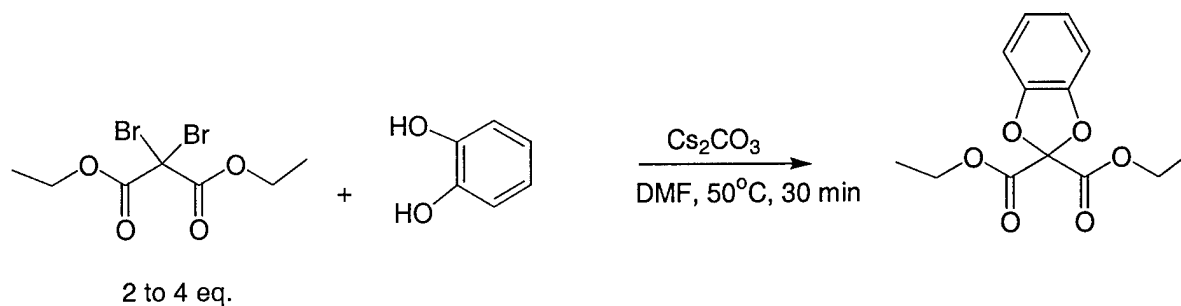
The development of a fluorescent probe for catechol estrogens (Task 2) is still ongoing. A three-step process involving ketal formation, hydrolysis, and re-esterification was successful with catechol. This process will be conducted on CE to determine the limits of detection. Synthesis of new α,α -dibromomalonamide probe has been conducted, and the use of this probe in a one-step derivation procedure is currently in progress. Once the appropriate fluorescent probe has been developed, it will be applied to the extraction procedure in Task 1 to test limits of detection in biological samples.

The development of an analytical assay capable of detecting CE-DNA adducts, CE, and MPEM at the low picomolar to high femtomolar level is necessary in order to detect these compounds at physiological levels. When completed, the analysis of human breast tissue will determine if a link between the production of CE-DNA adducts and the development of breast cancer exist. This would lead to a new biomarker for the early detection of breast cancer.

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APPENDICES:**Figure 1. Structure used to test extraction procedure.****Figure 2. Structural features of a successful fluorescent probe specific for the catechol moiety.****Figure 3. Reaction of diethyl α,α -dibromomalonate with catechol.**

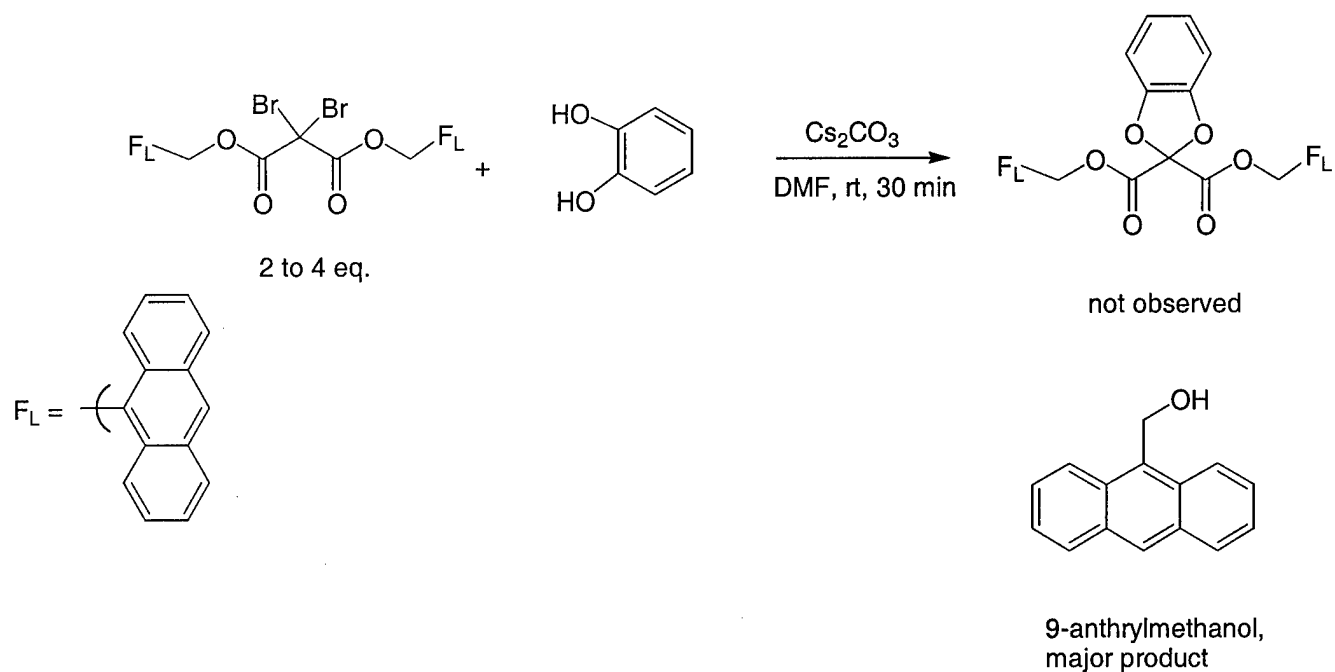


Figure 4. Reaction of catechol with bis-(9-methylanthryl) α,α -dibromomalonate.

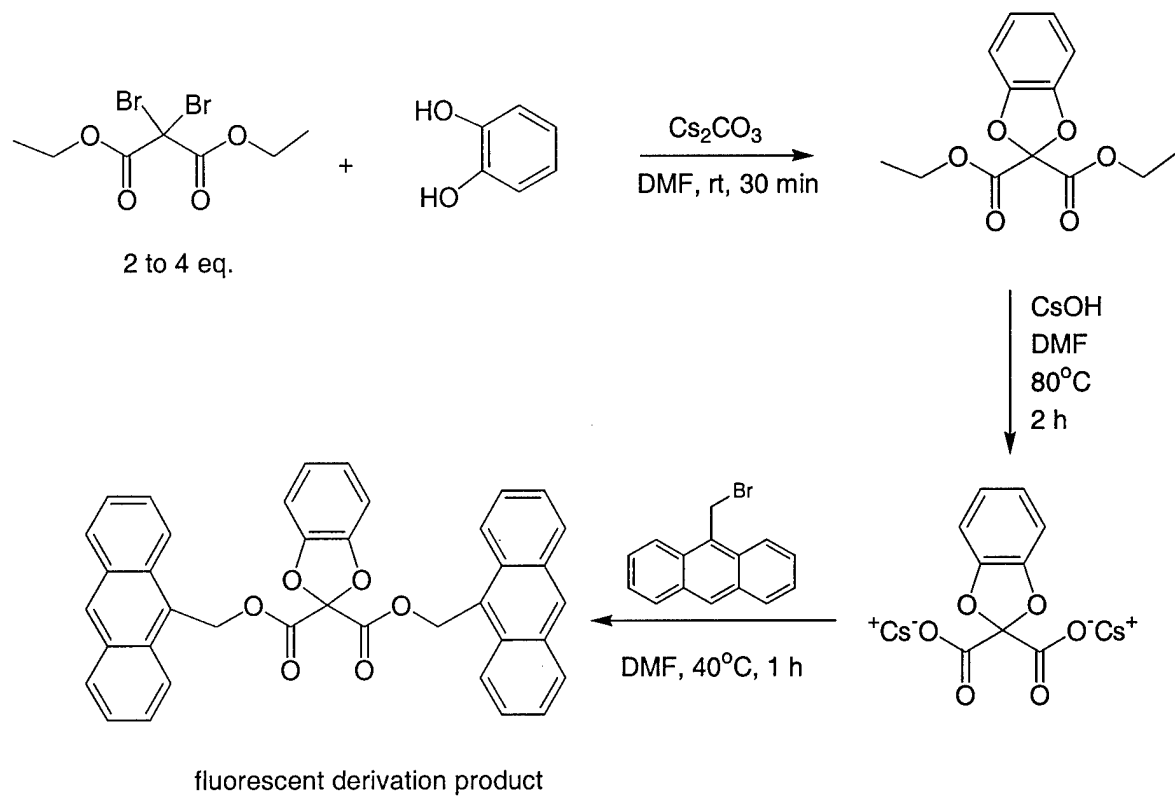


Figure 5. Three-step process for fluorescence labeling of catechols.

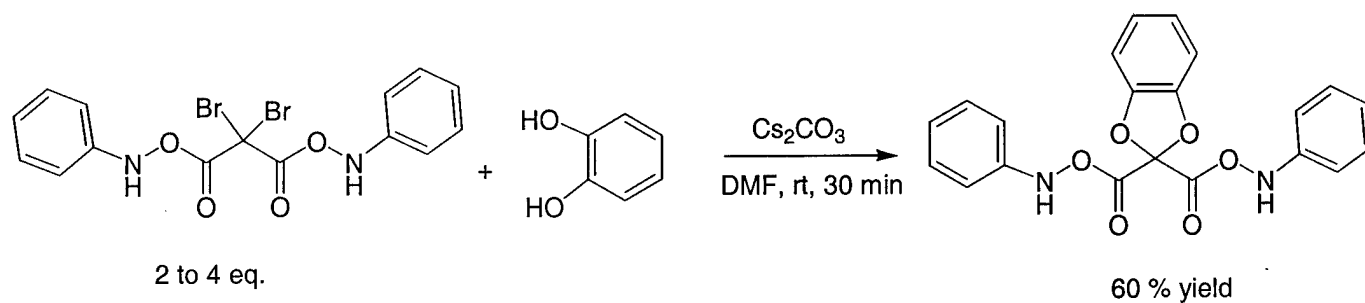


Figure 6. Reaction of catechol with bis-(N-phenyl) α,α -dibromomalonamide.

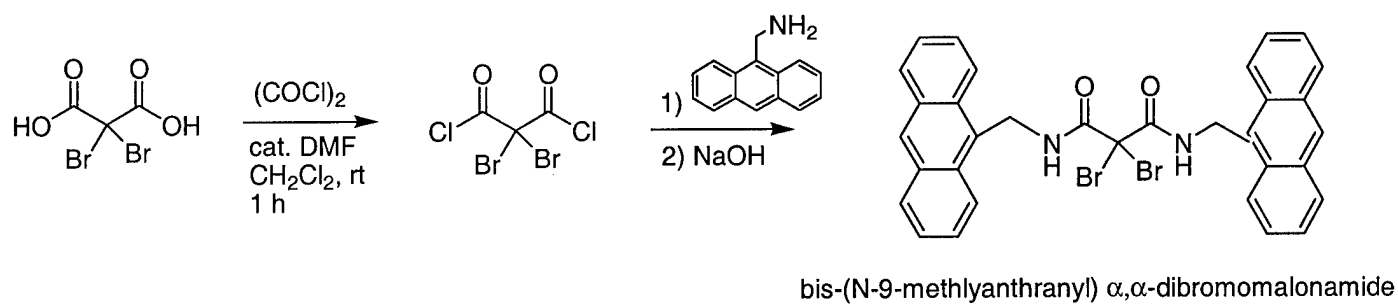


Figure 7. Synthesis of bis-(N-9-methylanthranyl) α,α -dibromomalonamide